Transcriptomic insights into the role of the spleen in a mouse model of Wiskott-Aldrich syndrome

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Abstract. Wiskott-Aldrich syndrome (WAS) is a rare X-linked primary immunodeficiency characterized by microthrombocytopenia, eczema, recurrent infection and increased incidence of autoimmune disorders and malignancy. WAS is caused by mutations in the *was* gene, which is expressed exclusively in hematopoietic cells; the spleen serves an important role in hematopoiesis and red blood cell clearance. However, to the best of our knowledge, detailed comparative analysis of the spleen between WASp-knockout (WAS-KO) and wild-type (WT) mice, particularly at the transcriptomic level, have not been reported. The present study investigated the differences in the transcriptomes of spleen tissue of 10-week-old WAS-KO mice. Comparison of the gene expression profiles of WAS-KO and WT mice revealed 1,964 differentially expressed genes (DEGs). Among these genes, 996 DEGs were upregulated and 968 were downregulated in WAS-KO mice. To determine the functions of DEGs, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses were performed for significantly upregulated and downregulated DEGs. The results showed that the levels of cell senescence and apoptosis-associated genes were increased, antigen processing and presentation mechanisms involved in the immune response were damaged and signal transduction processes were impaired in the spleen of WAS-KO mice. Thus, *was* gene deletion may lead to anemia and hemolysis-associated disease, primarily due to increased osmotic fragility of red blood cells, low hemoglobin and increased bilirubin levels and serum ferritin. These results indicated that senescence and apoptosis of blood cells also play an important role in the occurrence of WAS. Therefore, the present findings provide a theoretical basis for further study to improve the treatment of WAS.

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Introduction

Wiskott-Aldrich Syndrome (WAS) is a rare X-linked primary immunodeficiency disorder that affects males with a frequency of one in 10,000,000 (1). It can lead to recurrent infection, thrombocytopenia, eczema, a high incidence of malignancy and autoimmune complications (in 40-70% of patients) (2). These complications can be fatal if not treated with bone marrow transplantation or gene therapy (3). WAS is caused by a coding mutation in the *was* gene, which is located on the short arm of the X chromosome and was identified by positional cloning in 1994 (4). The *was* gene contains 12 exons and encodes a highly proline-rich protein of 502 amino acid residues, which is named WAS protein (WASp) (5).

The WASp protein contains multiple functional domains and serves a key role in the regulation of branched actin chain polymerization, primarily regulating the actin cytoskeleton in hematopoietic cells (6). The role of WASp deficiency in the development of autoimmunity in WAS has been explored extensively (7-9). It has been demonstrated that WASp is involved in regulation of immunity, cell proliferation, differentiation, activation and function in an actin-dependent manner (10,11). WASP also influences the immune response by regulating the transcription levels of CD19 receptor in B cells and inflammatory factors (12). Moreover, it has been demonstrated that WASp-deficient natural regulatory T cells are defective in suppressing effector T cells and B lymphocyte proliferation (13,14). However, the cellular and molecular mechanisms in WASp underlying WAS, particularly the pathogenesis of autoimmunity, remain unclear.

Studies have found that IL-10 production of regulatory B cells is decreased in WASp-knockout (WAS-KO) mice (15) and the abundance of Th1 and Th17 cells is increased (16), which leads to impairment of immune function. In addition, WASP-deficient T cells in WAS-KO mice show markedly impaired proliferation and antigen receptor cap formation in response to anti-CD3ε stimulation, which is consistent with patients with WAS (17,18). The spleen is the largest secondary lymphoid organ in the body and serves a major role not only in immunological functions but also in hematopoiesis and red blood cell clearance (19,20). Therefore, the spleen may serve an important role in the progression or treatment of patients with WAS and the study of the role of the spleen in autoimmunity in

WAS-KO mice may inform understanding of the pathogenesis of autoimmune manifestations in WAS.

The present study investigated the differences in the spleen transcriptome of 10-week-old WAS-KO and wild-type (WT) mice. Differentially expressed genes (DEGs) that were significantly altered were identified and Gene Ontology (GO) analysis to determine the specific functions of genes and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis to evaluate the enrichment of gene sets was performed. In addition, DisGeNET and Reactome analyses were performed to find possible novel autoimmune complications. To the best of our knowledge, the present study is the first to investigate transcriptome sequences in the spleen of WAS-KO mice, which may facilitate understanding of the cellular and molecular mechanisms in WAS.

Materials and methods

Mice and tissue processing. Was $^{-/-}$ (129S6/SvEvTac-Was^{tm1Sbs}/J; WAS-KO) and 129S6/SvEvTac (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). It is a mature model widely used in the study of WAS (21,22). The total number of WAS-KO mice is 6 and WT mice is 6. All mice were male used in this study. All animals were maintained with food and water *ad libitum* under a 12/12-h light/dark cycle at 22±2˚C, with 50±10% relative humidity. Ten-week-old male mice (about 25 g) were used and all experiments were approved by the Medical Ethics Committee of The Third People's Hospital of Shenzhen (approval no. 2021038). The mice were anesthetized with pentobarbital sodium (1%, 30 mg/kg, i.p.) and the spleen was isolated. Mice were euthanized by CO_2 gas (4.5 l/min) with 30% air displacement rate in an individually caging system (KW-AL-G; Nanjing Calvin Biotechnology Co., Ltd.). Total RNA was extracted from the right spleen using an RNAqueous™ kit (cat. no. AM1912; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Purity was determined by a NanoDrop 1000 (Thermo Fisher Scientific, Inc.).

RNA-sequencing (seq) analysis. RNA-seq library was prepared by a TruSeq RNA sample preparation kit (Illumina, Inc.) and sequencing of libraries was conducted on an Illumina HiSeq system. A total of three samples were collected from each group. The read depth was 30-50x10^6 bp for each sample. Skewer (v0.2.2) software (sourceforge.net) was used to dynamically remove joint sequence fragments and low-quality fragments from the 3' end of the sequencing data. FastQC (v0.11.5) software (Babraham Bioinformatics) was used to conduct quality control analysis on the preprocessed data. Sequence data were mapped to the mouse reference genome GRCm38 (23) with STAR (v2.5.3a) software (github.com). StringTie (v1.3.1c) software (ccb.jhu.edu) was used to calculate the original sequence count of known genes for all samples and the expression levels of known genes were calculated using fragments per kilobase of transcript per million fragments mapped.

DEG analysis. DEG analysis was conducted between WT and WAS-KO mice to identify significantly up- or downregulated genes. DEGs were assessed by DESeq 2 (v1.16.1) software (bioconductor.org). Benjamini-Hochberg multiple test correction method was used. DEGs were chosen according to $|\log_2$ fold-change $|\geq 1$ and adjusted P-value <0.05. Functional annotation clustering was performed using TopGO (v2.48) software (bioconductor.org). GO (geneontology.org) and KEGG (genome.jp) annotations were tested for enrichment and a Benjamini-Hochberg-corrected P-value <0.05 was considered to indicate a statistically significant difference. Functional analysis of disease annotation was performed via functional annotation and classification of disease types in the DisGeNET disease database (disgenet.org) for DEGs. Reactome metabolic pathway analysis was used to annotate DEGs for metabolic pathways in the Reactome database (reactome.org/). Heatmaps were plotted by Bioinformatics (bioinformatics.com.cn), a free online platform for data analysis and visualization.

Results

DEG analysis. DEGs were selected according to the criteria |log2 (fold-change) |≥1 and P<0.05. DEGs are shown in Fig. 1A. A total of 1,964 genes were differentially expressed in WAS-KO compared with WT mice, with 996 genes upregulated and 968 genes downregulated (Table SI). Cluster analysis of DEGs between the WT and WAS-KO groups was performed and the results are shown as a heatmap (Fig. 1B).

GO enrichment analysis. To determine the functions of the identified DEGs, GO enrichment analysis was performed for significantly upregulated and downregulated DEGs (Fig. 2). The top 10 biological processes (BP), cellular components (CC) and molecular functions (MF) in the GO analysis were selected. The GO results showed that upregulated DEGs were involved in 'heme metabolic process', 'cell division' and 'cofactor biosynthetic process', according to BP. In terms of CC annotations, 'cytoplasm', 'extracellular region part' and 'chromosome, centromeric region' showed significant enrichment. In terms of the MF annotations, 'cytoskeletal protein binding', 'ATPase regulator activity' and 'organic acid transmembrane transporter activity' showed significant enrichment (Fig. 2A). To determine the association between GO clusters, network analysis of the top 20 GO clusters in the upregulated DEGs was performed. Genes were enriched in 'erythrocyte development', 'cell cycle' and 'heme and pigment metabolic process' (Fig. 2B).

GO analysis of downregulated DEGs showed that they were involved in 'microtubule bundle formation', 'anatomical structure formation involved in morphogenesis' and 'cellular component assembly involved in morphogenesis' BPs. In terms of CC annotation, 'extracellular matrix component' and 'extrinsic component of membrane' showed significant enrichment. In terms of the MF annotation, 'ion binding', 'heparin binding' and 'glycosaminoglycan binding' showed significant enrichment (Fig. 2C). The network analysis of the top 20 GO clusters in the downregulated DEGs yielded four clusters: 'Anatomical structure morphogenesis', 'renal system', 'extracellular matrix' and 'cellular component assembly' (Fig. 2D).

KEGG analysis. To determine which pathways may be directly affected by gene deficiency in WAS-KO mice, the significantly upregulated and downregulated DEGs were analyzed using the KEGG pathway database. The significantly upregulated

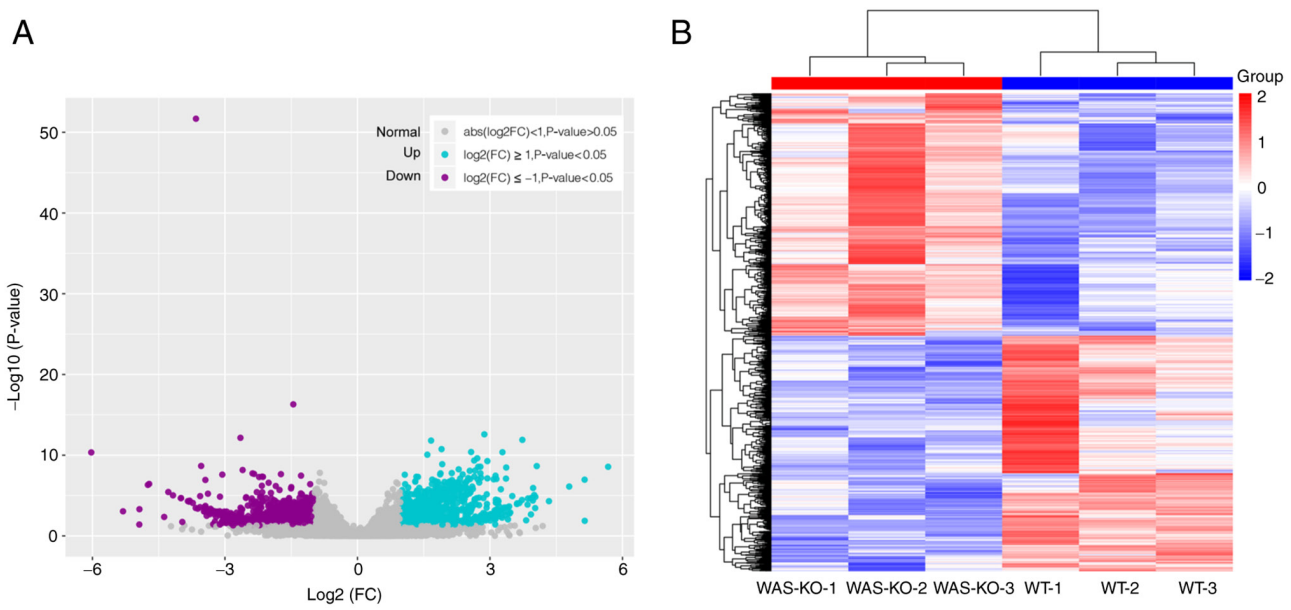

Figure 1. Volcano plot and heatmap of significant DEGs. (A) DEGs between WT and WAS-KO mice included 1,964 transcripts that were significantly altered (P<0.05, |log2 FC|≥1), with 996 upregulated and 968 downregulated transcripts. (B) DEGs between the WT and WAS-KO groups were cluster analyzed and the results are shown as heatmaps. DEG, differentially expressed gene; WT, wild-type; WAS-KO, Wiskott-Aldrich syndrome knockout; FC, fold-change.

DEGs were primarily involved in signaling pathways including 'cellular senescence', 'p53 signaling pathway' and 'ferroptosis' (a cell death pathway). A number of other DEGs were found to participate in different metabolic pathways, such as 'glutathione metabolism', 'glycolysis/gluconeogenesis' and 'glycerophospholipid metabolism' (Fig. 3A). To clarify the links between these pathways, a network analysis of the top 20 KEGG clusters in the upregulated DEGs was performed; the primary upregulated pathway was cell cycle and cell death (Fig. 3B).

KEGG results of downregulated DEGs showed that these genes were involved in 'platelet activation', 'Th1 and Th2 cell differentiation' and 'antigen processing and presentation' for the immune system and 'MAPK signaling pathway', 'calcium signaling pathway' and 'cAMP signaling pathway' for signal transduction (Fig. 3C). In addition, the network analysis of the top 20 KEGG clusters in the downregulated DEGs showed that the main downregulated pathway was signal transduction.

DisGeNET and Reactome analysis. To investigate potential symptoms caused by DEGs, a disease enrichment analysis was performed using the DisGeNET database. Analysis of the top 30 clusters of disease terms showed that 'anemia, hemolytic', 'anemia, sickle cell', 'hereditary spherocytic hemolytic', 'spherocytosis', 'thalassemia' and 'hemoglobin H disease' were highly enriched (Fig. 4A). To determine the association between these diseases, a network analysis of the top 20 clusters was performed. The results showed that anemia and hemolysis were primarily associated with 'serum ferritin increased', 'hemoglobin low', 'increased bilirubin level' and 'increased red cell osmotic fragility' (Fig. 4B).

To elucidate the mechanisms by which these diseases may occur in WAS-KO mice, enrichment analysis was performed using the Reactome database. Analysis of the top 30 clusters of Reactome terms showed that 'heme biosynthesis', 'activation of ATR in response to replication stress', 'collagen degradation', 'G1-TP53 regulates transcription of genes involved in G1 cell cycle arrest' and 'glutathione synthesis and recycling' were highly enriched (Fig. 4C). The network analysis of the top 20 clusters showed that 'collagen biosynthesis and modifying enzymes' and 'erythrocytes take up oxygen and release carbon dioxide' were associated with these terms (Fig. 4D).

Prognostic implications of derived markers genes in WAS. To validate the aforementioned signaling pathways and determine potential novel marker genes in WAS, expression levels of marker genes were compared between WT and WAS-KO mice (Fig. 5). Marker genes involved in CC assembly and morphogenesis were significantly decreased in spleen of WAS-KO mice, such as *Ccdc114*, *Ccdc136* and *Dnah1* (Fig. 5A). In addition, expression of apoptosis marker genes such as *Bcl2*, *Fth*, *Pdcd* and *Maged* increased significantly. Furthermore, the marker genes involved in the immune system, such as *Mapk*, *Rasgrp2*, *Plag4b*, *COL1A2* and *Hspa* were significantly downregulated. Moreover, the expression levels of marker genes involved in signal transmission, such as *Camk2b*, *Camk4*, *Rac1* and *Fos*, were significantly decreased in the spleen of WAS-KO mice. The information of these marker genes is shown in Table SII.

Discussion

WAS has a wide clinical spectrum ranging from mild with thrombocytopenia, recurrent infections and eczema to severe presentation, which can include complications such as life-threatening hemorrhage, immunodeficiency, atopy, autoimmunity and cancer (4,6). The pathophysiology of features of WAS is being elucidated by clinical and basic studies but remains unclear, which hinders the application of targeted therapies. Gene expression analysis is a useful tool in the

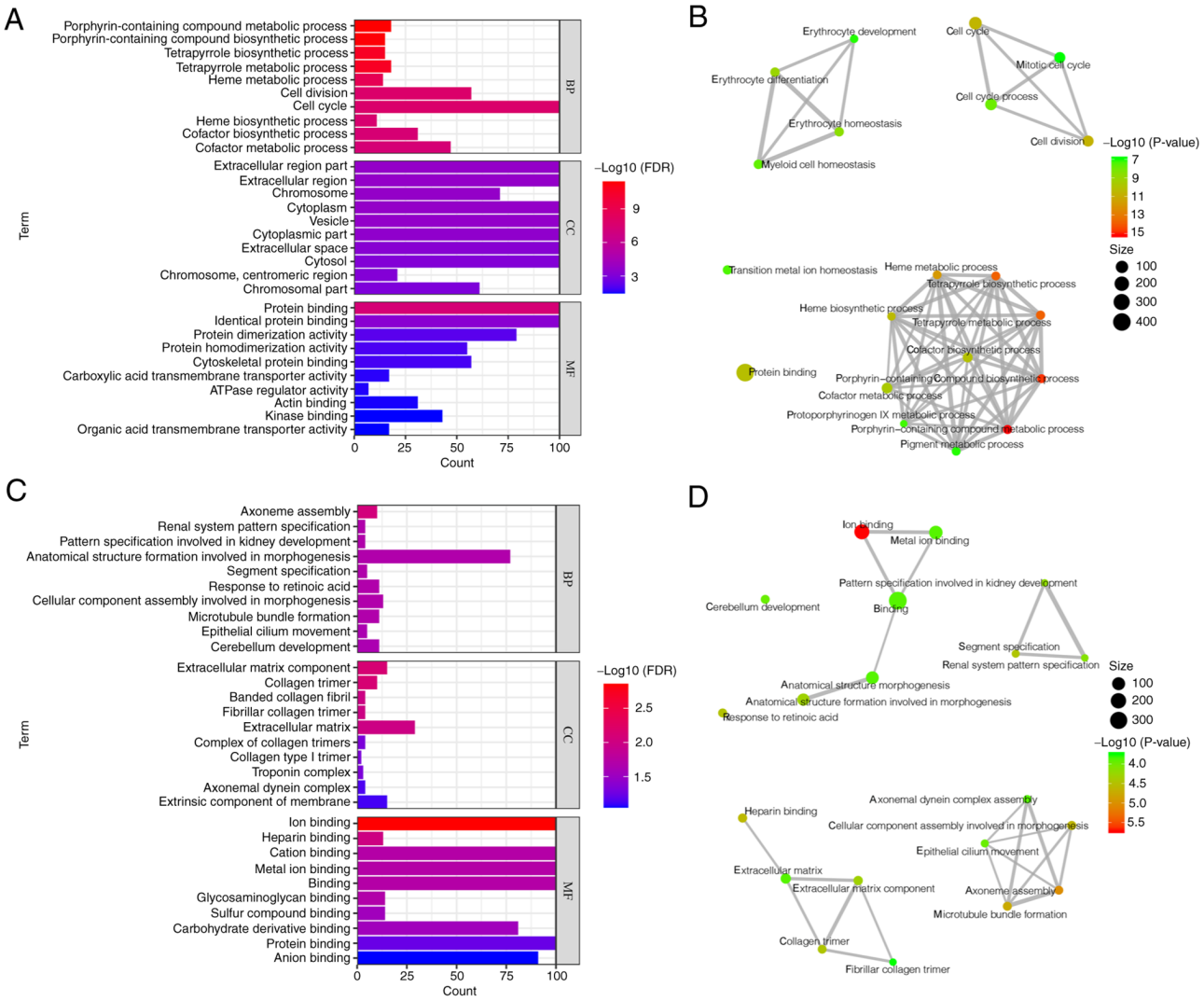

Figure 2. GO identifiers in the cluster of overlapping DEGs in WAS-KO and WT mice. (A) Top GO identifiers in the cluster of overlapping upregulated DEGs in WAS-KO and WT mice. (B) Network analysis of the top 20 GO clusters in the upregulated DEGs. (C) Top GO identifiers in the cluster of overlapping downregulated DEGs in WAS-KO and WT mice. (D) Network analysis of the top 20 GO clusters in the downregulated DEGs. BP, biological process; CC, cellular component; MF, molecular function; GO, Gene Ontology; DEG, differentially expressed gene; WT, wild-type; WAS-KO, Wiskott-Aldrich syndrome knockout; FDR, false discovery rate.

study of disease. The present study investigated the differences in spleen transcriptomes of 10-week-old WAS-KO and WT mice. The processes of heme metabolism, cell division and ATPase regulator activity were enhanced, but microtubule bundle formation and anatomical structure formation involved in morphogenesis were impaired in the spleens of WAS-KO mice. The levels of cell senescence and apoptosis-associated genes were increased, antigen processing and presentation mechanisms involved in the immune response were damaged and signal transduction processes were impaired in the spleens of WAS-KO mice. Gene deletion may lead to anemia and hemolysis-associated disease, mainly due to increased erythrocyte osmotic fragility, low hemoglobin and increased bilirubin levels and serum ferritin. These results indicated that the senescence and apoptosis of blood cells play a key role in the occurrence of WAS. However, most studies have focused only on the immune response (24-26). Therefore, the present findings may provide a broader theoretical basis for further study to improve the treatment of WAS.

The large amount of cell senescence and apoptosis in the spleens of WAS-KO mice promoted cell division and cofactor biosynthesis processes, but dysfunction in cell structure formation and microtubule bundle formation may lead to functional defects in newly generated cells and ultimately cause splenic damage spleen in these mice. Rawlings *et al* (27) examined the susceptibility to apoptosis of resting primary lymphocytes isolated from patients with WAS in the absence of exogenous apoptogenic stimulation. Rengan *et al* (28) also found accelerated cell death in WAS lymphocytes, as evidenced by increased caspase-3 activity. This suggests that *was* gene deletion leads not only to lymphocyte apoptosis but also to erythrocyte senescence and apoptosis; this topic requires more research. Here, marker genes of apoptosis, such as *Bcl2*, *Fth*, *PD* and *Mage* increased significantly in spleen of WAS-KO mice. Deletion of *was* gene leads to the differentiation defect of T cells by downregulating transcription level of *PD-1* and *Bcl-6*. (29,30). Hashimoto *et al* (31) found that *PD-1* and *Mage-a4* are involved in the aggressive elements of soft tissue

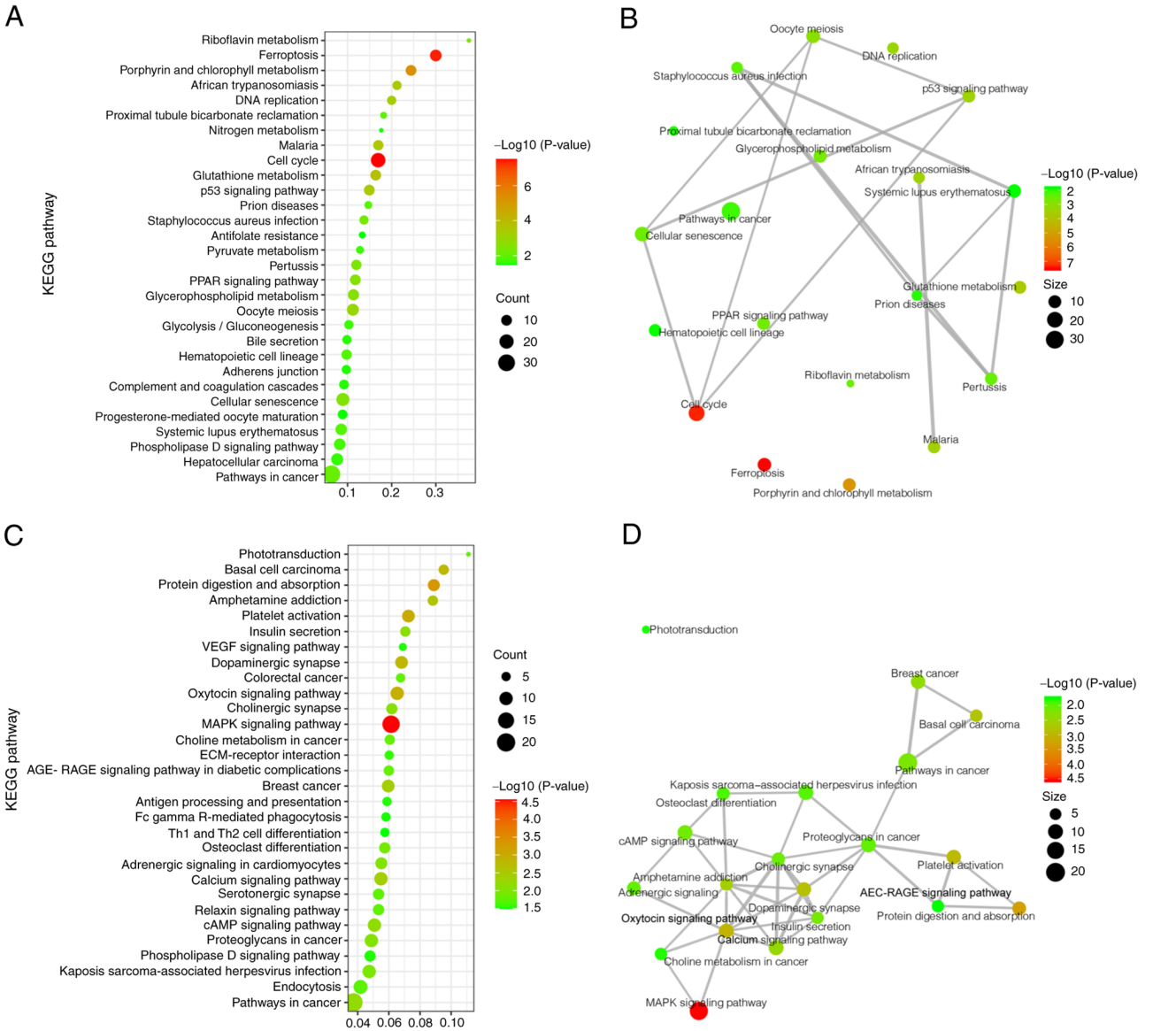

Figure 3. KEGG pathway analysis of DEGs in WAS-KO and WT mice. (A) Top KEGG pathways in the cluster of upregulated DEGs in WAS-KO and WT mice. (B) Network analysis of the top 20 KEGG pathways in the upregulated DEGs. (C) Top KEGG pathways in the cluster of downregulated DEGs in WAS-KO and WT mice. (D) Network analysis of the top 20 KEGG pathways in downregulated DEGs. DEG, differentially expressed gene; WT, wild-type; WAS-KO, Wiskott-Aldrich syndrome knockout; KEGG, Kyoto Encyclopedia of Genes and Genomes.

sarcomas (32). In addition, ferroptosis is an iron-dependent programmed cell death event, which affected by *Fth* gene (33). To the best of our knowledge, there is no research on the correlation between *Fth* and WAS. Thus, the marker genes *PD*, *Mage* and *Fth* identified here may affect the progression of WAS. In addition, reactive oxygen species (ROS) and inflammation may induce cell death (34,35). Production of ROS is associated with dynamic actin cytoskeleton reorganization (36) and NADPH oxidase-dependent physiologically generated ROS negatively regulate actin polymerization in stimulated neutrophils via driving reversible actin glutathionylation (37). Therefore, neutrophil dysfunction induced by ROS may accelerate pathogenesis of WAS. Furthermore, inflammation is involved in immunodeficiency (38). However, no signaling pathways directly associated with ROS or inflammation were enriched in the present study. The roles of ROS and inflammation in WAS should be investigated in future.

WASp is a cytoskeletal scaffolding adapter that coordinates transmission of stimulatory signals to downstream inducers of actin remodeling and cytoskeletal-dependent T cell responses (39). To the best of our knowledge, however, there is a lack of studies on the interaction between marker genes in the immune system and WAS. The present study found marker genes involved in immune system such as *Mapk*, *Rasgrp2*, *Plag4b*, *COL1A2* and *Hspa*, which may contribute to the study of WAS. Heat shock proteins serve an important cytoprotective role in cells exposed to stressful conditions and are implicated in auto-immune disease (40,41). In addition, MAPK signaling has roles both in innate and adaptive immune responses, including induction of pro-inflammatory mediators (42,43). T-cell receptors, upon binding to specific ligands of major histocompatibility complex (MHC) molecules on antigen-presenting cells, initiate intracellular signaling that leads to extensive actin polymerization (14,44). Thus, marker

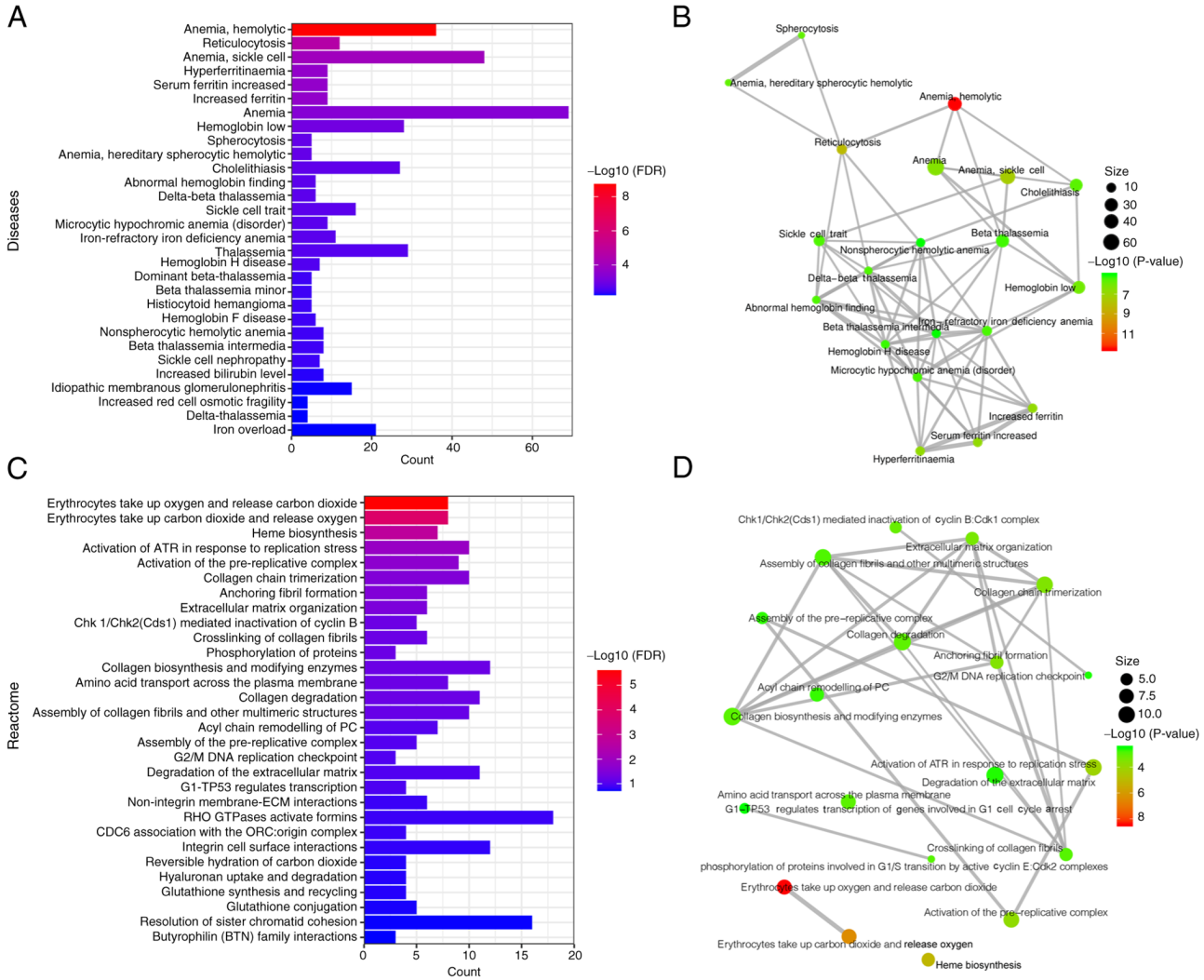

Figure 4. DisGeNET and Reactome analyses of DEGs in WAS-KO and WT mice. (A) Top disease analyses in the cluster of DEGs in WAS-KO and WT mice. (B) Network analysis of the top 20 DEGs. (C) Top Reactome analysis in the cluster of DEGs in WAS-KO and WT mice. (D) Network analysis of the top 20 Reactome analyses in DEGs. DEG, differentially expressed gene; WT, wild-type; WAS-KO, Wiskott-Aldrich syndrome knockout; FDR, false discovery rate.

genes belonging to MHC type, such as *H2-Q6*, *H2-T10* and *H2-T10*, may be associated with actin polymerization. Furthermore, WAS-interacting protein influences the function of CD19 as a general hub for PI3K signaling by regulating the cortical actin cytoskeleton in humans with WAS (45). Consistent with this, marker gene *pik3r5*, which is involved in PI3K signaling pathway, was downregulated in WAS-KO mice, suggesting that *pik3r5* is a potential marker gene for WAS.

In addition, WASp serves an essential role in signal transduction and effector functions of T cells; signal transduction regulating the function of T cells in immune response is impaired in WAS (14). In the present study, the expression levels of marker genes involved in signal transmission, such as *Camk2b*, *Camk4*, *Rac1* and *Fos*, were significantly decreased in the spleen of WAS-KO mice, suggesting the signal transduction in WAS-KO mice was impaired. For signal transduction, there are four pathways required, of which MAPK pathway is the most important (46). The Ras pathway activates the extracellular receptor-activated kinase (Erk) by phosphorylating its substrate, Elk1. Phospho-Elk1 then stimulates the transcription of c-Fos, a component of the transcription factor activation protein 1 (47). WASp is essential for nuclear translocation of phospho-Erk, Elk1 phosphorylation and expression of c-Fos (48). This suggests that *Ras1* and *Fos* may be used as clinical markers to evaluate Ras signaling pathway in WAS. Furthermore, T cell receptor engagement triggers mobilization of Ca^{2+}, which is required for T cell activation, gene expression, motility, synapse formation, cytotoxicity, development and differentiation (49,50). T cells from patients with WAS and WAS-KO mice show defects in intracellular Ca^{2+} mobilization (51). Here, marker genes *Camk2b* and *Camk4* were significantly downregulated in spleen of WAS-KO mice and may be involved in the decreased intracellular Ca^{2+} mobilization. Therefore, the present results may improve the knowledge of signal transduction in WAS. Some of these marker genes have been confirmed to be involved in the occurrence of WAS by clinical testing (52,53), which indicates that the marker genes detected in this study have the potential for clinical application.

Studies have found hemolysis and thrombocytopenia in WAS (54,55); for example, Burroughs *et al* (56) found that hematopoietic cell transplantation (HCT) significantly

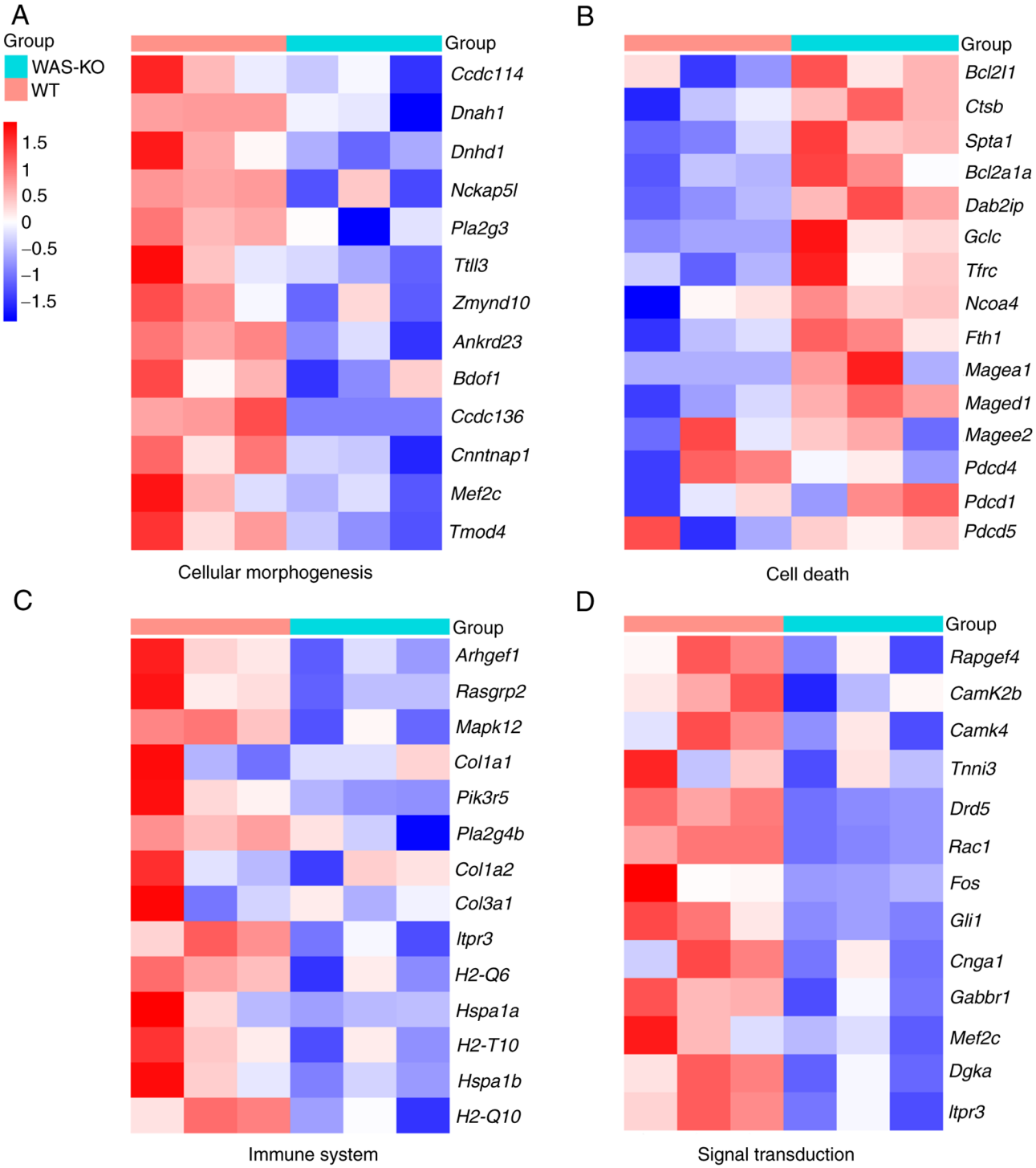

Figure 5. Relative expression of marker genes in WAS. Heatmap of differentially expressed marker genes involved in (A) cellular morphogenesis, (B) cell death, (C) immune system and (D) signal transduction. Expression is showed with $\log_{10}$(fragments per kilobase of transcript per million fragments mapped). WT, wild-type; WAS-KO, Wiskott-Aldrich syndrome knockout.

increases platelet levels in patients with WAS and is associated with better outcomes when performed at a younger age. The present study found that thrombocytopenia may be associated with increased erythrocyte osmotic fragility, low hemoglobin and increased bilirubin levels and serum ferritin, which may be targeted by gene therapy. Moreover, Rohrer *et al* (57) found WAS in a family with Fanconi anemia, indicating the occurrence of these two rare genetic disorders in a single family or the existence of an unusual variant of Fanconi anemia. This is consistent with the present prediction by disease enrichment analysis that *was* gene deletion may lead to anemia- and hemolysis-associated disease.

The present study detected 1,964 DEGs between WAS-KO and WT mice but only showed the top 30 enrichment results; other results indicated that the role of the spleen in WAS needs more attention in further studies (data not shown). In addition, the present study did not verify expression changes of DEGs by other methods. Further analysis of transcriptome

data available may aid in discovering novel mechanisms to improve therapies for WAS, especially in the context of anemia and primary immunodeficiency. Moreover, clinical samples are being collected from patients with WAS; analysis of differences in transcriptome levels between WAS-KO mice and patients with WAS in future studies and identification of marker genes may provide the basis for clinical gene therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The RNA-seq data used in this manuscript are publicly available in the Gene Expression Omnibus repository (accession no. GSE214745; ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE214745).

Authors' contributions

FFL, JY, QG, YX, LLW, YYH and CP contributed to the study design. FFL, JY and CP analyzed the data. QG, YX, LLW and YYH collected data. FFL wrote the manuscript. CP revised the manuscript. CP and FFL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved by the Medical Ethics Committee of The Third People's Hospital of Shenzhen (approval no. 2021038).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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